Long-Term Cultivation of Canine Keratinocytes*

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The growth characteristics and morphology of canine keratinocytes grown in vitro were studied. Keratinocytes from canine oral mucosa, ear skin, and ventral abdominal skin were grown in culture either as explants or as dispase/trypsin-derived suspensions in the absence of a feeder cell layer. Cholera toxin and epidermal growth factor were essential to the successful long-term growth and propagation of the cells during multiple passages. Keratinocytes from all tissue sources, either as primary cultures or subcultivated for up to 10 passages, had growth characteristics and morphology similar to that reported in other species. The use of cultured canine keratinocytes should provide a suitable model for comparative *in vitro* studies of the pathogenesis of dermatologic diseases. *J Invest Dermatol 88: 202–206, 1987*

The dog has been widely used as an animal model of numerous human diseases including a variety of dermatologic disorders [1]. Many inflammatory, neoplastic, proliferative, and immune-mediated skin disorders of humans have counterparts in the dog [2,3]. However, the biologic, morphologic, biochemical, and immunologic characteristics of canine stratified squamous epithelium (SSE) grown in vitro has not been well defined. Since many investigations of pathogenetic mechanisms utilize in vitro studies, it is important that the characteristics of canine SSE in culture be defined if the dog is to be fully exploited as a model for such studies.

In the past decade a variety of techniques for short- and long-term in vitro cultivation of keratinocytes obtained from several species have been described [4–8]. Although a single report describes the use of canine keratinocytes derived from short-term cultures of canine epidermis in transplantation studies [9], the growth characteristics and morphology of canine keratinocytes grown in vitro have not been defined and long-term cultures have not been studied. The purpose of this report is to define the optimal experimental condition for obtaining and propagating long-term cultures of keratinocytes of canine origin and to determine the morphologic characteristics of these cells during serial passages.

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Abbreviations:

CT: cholera toxin
EBSS: Earle's balanced salt solution
EGF: epidermal growth factor
FCS: fetal calf serum
MEM: minimum essential medium
SSE: stratified squamous epithelium

MATERIALS AND METHODS

Materials Chemicals and supplies were obtained from the following sources: Earle's balanced salt solution (EBSS), calcium- and magnesium-free EBSS, minimum essential medium (MEM), chick plasma, chick embryo extract, trypsin, gentamicin, and antibiotic/antimycotic from Gibco, Grand Island, New York; HEPES, BES, TES, and Na_4 EDTA from Cal Biochem-Behring, San Diego, California; amphotercin B from Sigma, St. Louis, Missouri; cholera toxin (CT) from Schwartz-Mann, Orangeburg, New York; epidermal growth factor (EGF) from Collaborative Research, Lexington, Massachusetts; DMSO from Pierce Chemical, Rockford, Illinois; dispase (Grade II) from Boehringer Mannheim, Indianapolis, Indiana; and fetal calf serum (FCS) from Amour Pharmaceutical, Kankakee, Illinois. Several lots of FCS were screened and those that best supported the growth of canine keratinocytes were used.

Tissues Primary cultures of canine keratinocytes were established from canine oral mucosa, ear skin, and ventral abdominal skin. Tissues were obtained from either anesthetized dogs or from necropsy material within 2 h after death. The area of tissues to be sampled was shaved in the case of skin samples, and all tissues were washed twice with povidone iodine followed by 70% alcohol. Full-thickness skin was removed and placed in EBSS with 15 mM HEPES, 10 mM BES, 10 mM TES, pH 7.4, containing penicillin (1000 U/ml), streptomycin (1 mg/ml), and Fungizone (2.5 μg/ml). Excess dermal tissue was then removed by trimming.

Explants Explant cultures were established using slight modifications of the method of Jepsen, MacCallum, and Lillie [6]. Briefly, six 3 × 2 × 2 mm pieces of tissue were placed into individual plasma clots in 25-cm^2 plastic culture flasks (Corning). The clots were allowed to form for 30–40 min at room temperature prior to the addition of 5 ml of complete media consisting of MEM with Earle's salt mixture, 20% FCS, 50 μg/ml gentamicin, 2.5 μg/ml amphotercin B, and 0.5% (vol/vol) DMSO. Media were routinely changed 3 times weekly. All cultures were maintained at 34°C with 5% CO_2 in air.

Cell Suspensions Following removal of excess dermal tissues, 4 × 4 mm pieces of tissue were placed in complete media with 10 mg/ml dispase and incubated at 4°C for 18 h. The epidermis

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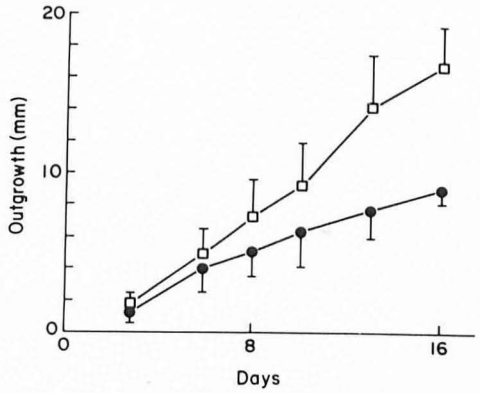


Figure 1. Effect of growth factors on keratinocyte outgrowth from explants. Each *point* represents 20 explants. *Open squares* = with 10 ng/ml EGF + 10^{-10} M CT. *Closed circles* = without growth factors.

was then easily removed from the dermis and placed in calcium- and magnesium-free EBSS containing 0.25% trypsin, 5 mM Na_4 EDTA, 15 mM HEPES, and 10 mM BES, 10 mM TES, pH 7.4 (trypsin–EBSS mixture). After 45-min incubation at 20°C, the cells were dispersed by pipetting. An equal volume of complete media was then added followed by centrifugation for 5 min at 700 *g*. The pellet was resuspended in complete media and 2.5 × 10^6 cells in 5 ml were plated onto 25-cm^2 tissue culture flasks. Cells were greater than 80% viable as assessed by trypan blue exclusion.

Subcultures When cultures reached confluence they were rinsed in calcium- and magnesium-free EBSS followed by incubation in trypsin (0.25%)–EBSS mixture. The trypsinized cells were then added to an equal volume of complete media, centrifuged, resuspended in complete media without DMSO and plated at 1–3 × 10^4 cells/cm^2. Cells were greater than 95% viable by trypan blue exclusion. At the second or third passage, the FCS was reduced to a 10% final concentration.

Growth Factors Due to the inability to maintain and expand long-term growth of canine keratinocyte cultures in basal media, the effects of growth factors were evaluated. For primary cultures, CT (10^{-10}M) was added continuously and EGF (10 ng/ml) was added to basal media beginning on day 3 [7]. The dose response for CT, EGF, and CT plus EGF was determined using cells at the first passage. Freshly trypsinized cells were washed in MEM with 10% FCS, centrifuged, and resuspended in MEM and 10% FCS with 10^{-11}, 10^{-10}, 10^{-9}, or 10^{-8} M CT, 1, 10, or 100 ng/ml EGF, 10^{-11} M CT and 1 ng/ml EGF, 10^{-10} M CT and 10 ng/ml EGF, or 10^{-9} M CT and 100 ng/ml EGF added. Cells were then plated at 1 × 10^4 cells/cm^2 in 48-well tissue culture plates. After 24 h, the media were removed and the wells washed twice in MEM to remove nonadherent cells. On days 3, 7, 10, and 14, duplicate wells from each treatment were trypsinized and the cells counted in a hemocytometer. Media in the remaining wells were changed on the same days. The experiment was repeated 3 times with 3 separate tissue sources.

Observations Cultures were examined 3 times weekly and photographed as appropriate using a Nikon inverted phase microscope.

Electron Microscopy Cultures were examined by electron microscopy following in situ fixation, dehydration, and embedding by routine methods [6].

RESULTS

Effects of Growth Factors Primary explant cultures were initially similar in the presence or absence of growth factors (Fig 1). Outgrowth of epithelial cells occurred in 2–4 days in either medium. However, in the absence of growth factors, canine keratinocytes grew at progressively slower rates ($p < 0.05$ after 8 days and thereafter). In 12 separate experiments involving 360 explants and 6 disassociated epithelial cultures, canine keratinocytes grown in MEM and FCS alone never grew beyond 2 passages. Those few cultures that did survive passage were subcultivated at 6 × 10^4 cells/cm^2. In 8 subsequent experiments utilizing both explants and suspension cultures in which 10^{-10} CT and 10 ng/ml EGF were added to the basal media, every culture has survived multiple passages (>5), and the 4 cultures thus far evaluated continued to

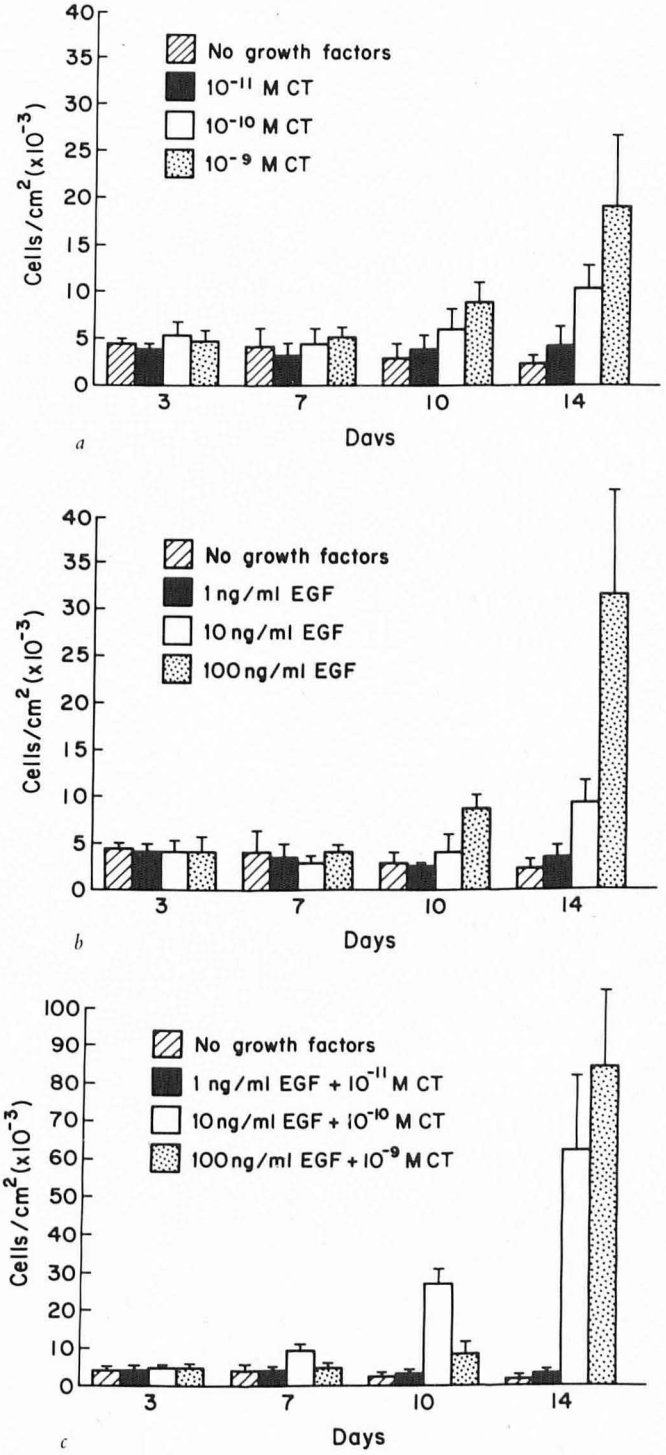


Figure 2. Dose-response curves for CT (*a*), EGF (*b*), and EGF + CT (*c*). Each *point* represents 6 wells.

grow after at least 10 passages with no observable change in growth rates or morphology. These cells are routinely subcultured at 1–3 × 10^4 cells/cm^2. At this initial cell density, cultures are confluent (>10^5 cells/cm^2) in 12–14 days.

The growth effects of various doses of CT, EGF, and CT plus EGF are shown in Fig 2. Although 100 ng/ml and 10 ng/ml EGF increase cell density by 10 and 14 days, respectively ($p < 0.05$), and 10^{-11} to 10^{-8} M CT increased cell numbers by 14 days ($p < 0.05$), the best growth occurred when CT and EGF were both included in the media. When CT and EGF were included at either 10^{-10} M CT and 10 ng/nl EGF or 10^{-9} M CT and 100 ng/ml EGF, significantly increased cell numbers were observed at 10 and 14 days relative to either growth factor alone. Although 10^{-8} M CT resulted in increased cell numbers at 14 days, the number of cells was less than that observed at 10^{-9} or 10^{-10} M CT ($p < 0.01$). In addition, many detached cells were present in the cultures with 10^{-8} M CT, the remaining cells were large and flattened, and the cells could not be successfully subcultivated. Maximal cell numbers at 14 days were obtained in media containing 10^{-9} M CT and 100 ng/ml EGF.

Phase Contrast Observation Epithelial cells began to grow out of explants by 2–3 days (Fig 2*a*). The polygonal cells near the explant became progressively more closely packed as the leading edge advanced. In a few explant cultures, fibroblasts were noted. This occurred more often and the extent of contamination was greater in the absence of growth factors and in tissues derived from young dogs. In one experiment using oral mucosal explants from an 8-day-old dog, 20 of 20 explants grown in the absence of growth factors had significant fibroblast outgrowth (>10% of outgrowth area at 7 days). Only 6 of 20 explants from the same source had significant fibroblast outgrowth when EGF (10 ng/ml) and CT (10^{-10} M) were included in the media. In tissues from adult dogs, significant fibroblast outgrowth was rare in the presence of EGF and CT (<2% of explants). In the presence of CT and EGF, the rapidly growing epithelium displaces the fibroblast from the surface of the plate (Fig 2*b*). In those cultures where a few fibroblasts remained, they were differentially removed by incubating the flask for 3–5 min in EBSS–trypsin mixture. The fibroblasts rapidly round up and float free from the surface while the epithelium remains firmly attached. These flasks were then either passaged at 3 × 10^5 cells/cm^2 or had fresh complete medium added. In all cases, these procedures resulted in the absence of any recognizable fibroblast in subsequent subcultures as determined by visual observation, light and electron microscopy, and immunocytochemical staining for keratin.

In dispase/trypsin-derived cultures, less than 1% of the cells attached to the culture plate individually or as small clumps within 24 h. By 3–5 days, a small percentage of the cells had begun to form colonies and by 21–28 days the colonies had coalesced to cover the entire surface of the plate in the hardiest cultures. However, in some cases proliferation markedly decreased and the cells assumed a large, polygonal squamous morphology. By 10–14 days only approximately 50% of the surface was covered by epithelium (Fig 3*c*). This percentage changed little if the cultures were left for 7–14 additional days. However, if cultures were passaged at this stage, they rapidly reentered a growth phase indistinguishable from that seen with subcultivated cells from primary explant cultures.

In subcultures, cells attached to the surface within a few hours. Cells subcultured at 1 × 10^5 cells/cm^2 were confluent within 2–3 days; subcultures plated at 3 × 10^4 cells/cm^2 were confluent in 10–12 days (Fig 3*d*). If such cultures were maintained without passage, opalescent areas similar to those seen in some primary cultures (Fig 3*c*) appeared which correspond to areas where large squamous cells cover the surface. These areas were maintained for periods of up to 8 weeks, and as cultures age, these areas predominate.

Electron Microscopy The ultrastructures of all primary cultures and subcultures were similar and although both often had the appearance of a monolayer (Fig 3*b,d*), ultrastructural examination demonstrated that differentiation and migration occurred simultaneously in the cultures. The cells were multilayered with a basal layer of squamous cells (Fig 4*a*). The basal cells have abundant mitochondria, numerous lysosomes, abundant free ribosomes and polyribosomes, a well-developed Golgi, a few clusters of endoplasmic reticulum, and keratin filaments either randomly aligned or in bundles in a perinuclear location (Fig 4*b*). The nuclei had one or more nucleoli, marginated heterochromatin, and were often deeply indented. Cells in all layers were attached by desmosomes and were separated by moderate intercellular spaces. In the intermediate layers, cells were flattened with elongate nuclei oriented parallel to the surface. Mitochondria and lysosomes were decreased in number while keratin filaments were more abundant. In the uppermost layers, organelles became sparse and the surface often had small, knob-like projectives.

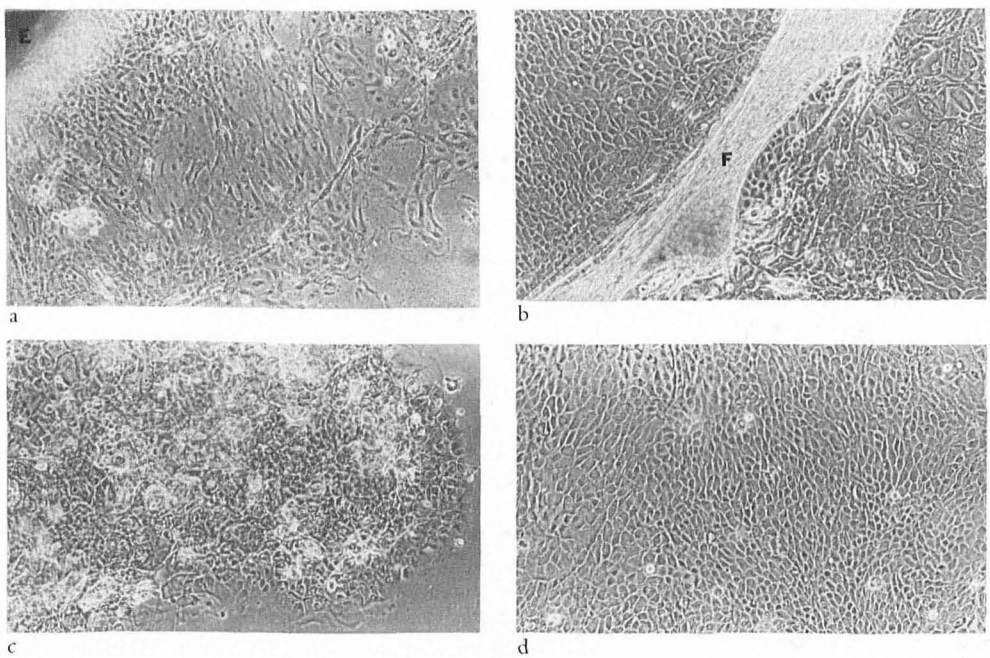
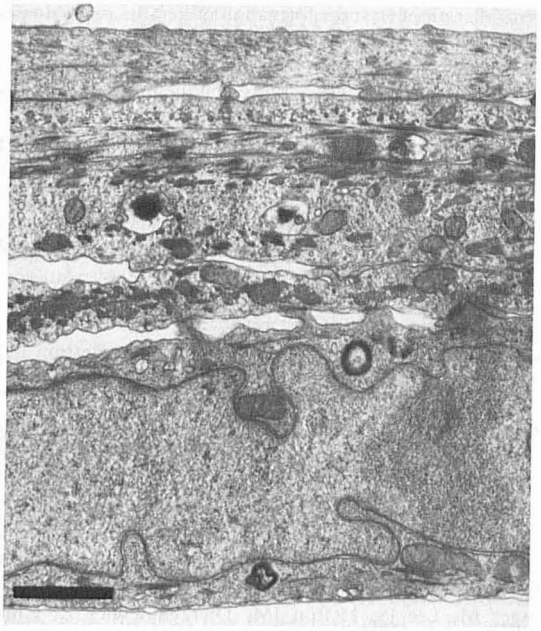


Figure 3. *a,* Primary explant of canine oral mucosa after 3 days in culture. *E* = Explant. *b,* Fibroblastic focus in a primary explant culture. Epithelial cells from 2 adjacent explants displace the fibroblasts from the surface causing them to pile up. *F* = Fibroblast. *c,* Dispase/trypsin-derived primary culture. The epithelium has begun to differentiate and proliferation has dramatically decreased prior to reaching confluence. *d,* Confluent cultures of canine keratinocytes. Cells were passaged for the fifth time at 1:3 14 days previously. *a–d* ×52.



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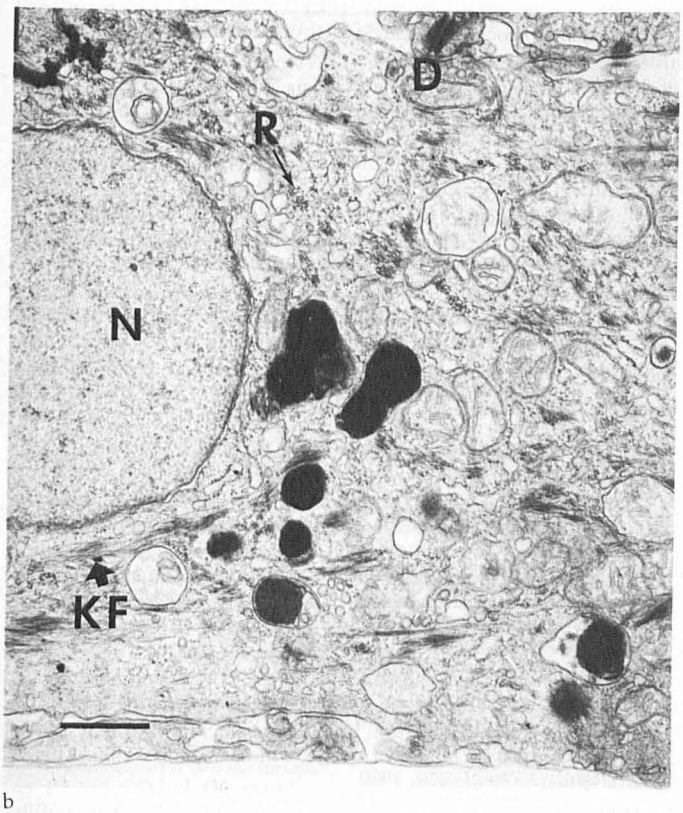

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Figure 4. *a*, Electron micrograph of a confluent primary explant culture. *Bar* = 1 μm. *b*, Higher magnification of a basal cell in primary culture. *N* = nucleus; *KF* = keratin filaments; *R* = ribosomes; *D* = desmosomes. Note also abundant mitochondria and lysosomes. *Bar* = 0.5 μm.

Effects of Tissue Source No differences in growth characteristics or morphology were noted among oral mucosa, ear skin, and ventral abdominal skin. Tissues taken from anesthetized dogs and dogs within 2 h of death were equally viable.

DISCUSSION

The purpose of these studies was to establish methods for growing and maintaining long-term cultures of canine keratinocytes and to define the growth characteristics and morphology of the cells over multiple passages. A variety of techniques have previously been used to establish primary keratinocyte cultures from several mammalian species (reviewed in [5]). In the studies presented here, using both explants and disaggregated epithelia to initiate primary cultures of canine keratinocytes from several tissue sources, the most consistent and easily reproducible results were obtained with explants.

The pattern, morphology, and rate of explant outgrowth was similar to that previously reported in the mouse [10] and humans [11–14]. The reason for the inconsistent growth of primary cultures of trypsin-disassociated cells is unknown but may relate to the absence of a feeder cell layer [15], the initial cell density, pH, or temperature [16]. Feeder cell layers have been shown to be important in establishing and maintaining human [7] and rat [15] keratinocytes derived from trypsinized skin. Eisenger et al [16] were able to grow human epidermal cells in the absence of dermal components when plates were seeded at a density similar to that which we used. However, in their system the cells were maintained at 35–37°C and the pH of the culture medium was at 5.6–5.8. Under these conditions with human cells, 70–80% attachment rates were observed with up to 20% of the cells being "basal" cells after 3 days. No growth was observed at the pH (7.4) used in our studies. They did, however, report a similar failure of colonies of human epidermal cells to coalesce under suboptimal conditions [16]. The ability to subculture the nonconfluent, apparently growth-arrested, canine cells in our studies, and obtain growth similar to that for growing cultures indicates that a population of basal cells capable of proliferation is present in these cultures. The reason for the observed shift from proliferation to differentiation in these primary cultures and the mechanism by which subcultivation reactivates the proliferative capacity is unknown. Marcelo et al [17] reported the successful growth of mouse keratinocytes derived from trypsinized epithelia in the absence of feeder cell layers. Although the seeding density was similar (2×10^4 cells/cm^2), the cell suspension was enriched for basal cells by discontinuous Ficoll gradient purification prior to initiation of the cultures. Thus, the initial cell density of keratinocytes capable of proliferation was higher than in our method.

The effects of CT and EGF on cultured keratinocytes have been studied extensively in the last decade. Both CT and EGF act directly [18,19] to increase the growth and life span of cultured keratinocytes [7,18–23]. Although Jepsen et al [6] have successfully subcultivated rat lingual epithelia in the presence of 20% FCS and the absence of feeder cells and growth factors, we found that CT and EGF were necessary for the sustained growth of canine keratinocytes. Eisenger et al [9] have previously shown that canine keratinocytes could grow for only 2–3 passages in the presence of hydrocortisone as the only growth supplement. The effects of EGF and CT were not evaluated in their studies. Cholera toxin is an 84,000 dalton protein composed of two subunits [19], which irreversibly activates adenylate cyclase via stimulation of the nucleotide regulatory component of the adenylate cyclase system [24]. All known effects of CT on keratinocyte growth are mediated by the resulting increase in cAMP [19]. Epidermal growth factor is a small protein with pleiotrophic cellular effects [25]. The exact mechanisms by which EGF enhances keratinocyte growth [20,21] are not clearly defined, nor is the relationship among EGF, cyclic nucleotides, and the cAMP-dependent protein kinase [26,27].

Cholera toxin is known to increase the growth rate [18–20], and alter the differentiation [22] of keratinocytes. Growth stimulation is maximal at lower cell densities [19,20], decreases at moderate cell densities, and CT may be inhibitory after cells are confluent [19]. Epidermal growth factor permits the successful subcultivation of cells, enhances colony-forming efficiency, and increases growth especially at high cell densities [20,21]. The combined effects of CT and EGF are enhanced colony formation (EGF and CT) [19,21], increased growth at low cell density (CT) [20,21], and increased growth at high cell density (EGF) [18]. These combined effects were clearly observed in our studies. At both 10 ng/ml EGF and 10^{-10} M CT or 100 ng/ml EGF and 10^{-10}

M CT, the growth was significantly greater than with either growth factor alone. Cholera toxin and EGF also increased the growth rate of primary explant cultures. Cultures are currently routinely grown in 10^{-10} M CT and 10 ng/ml EGF since these doses provide acceptable growth rates, and are well below the toxic dose of CT. Although EGF is reported to result in flattening of human keratinocytes [18] and CT is reported to have a similar effect on pig epithelia [23], no changes in morphology were observed in canine keratinocytes in any of the treatments used in these studies.

Since contaminating fibroblasts and feeder cells interfere with many biochemical studies of keratinocytes, we wanted to develop subcultures as free of contaminating cells as possible. By using dispase [20] to remove the epithelium prior to trypsinization in disaggregated primary cultures, fibroblast contamination was minimal. In explant cultures, fibroblast contamination was most pronounced in cultures grown in growth factor-free media and for tissues obtained from young dogs. These results agree with those reported for human keratinocyte cultures [20]. A variety of techniques have been used to remove contaminated fibroblasts from epidermal cultures [6,7,20,23,28,29]. The differential sensitivities of fibroblast to calcium-free media [7], and trypsin [6] combined with the ability of keratinocytes to displace fibroblasts from the surface [6,20] has been observed by others. Although a minimal number of fibroblasts could be present in our subcultures, no fibroblasts have been observed in thousands of cells stained for keratin or vimentin or examined by phase contrast, light, or electron microscopy.

The morphology of canine keratinocytes in culture is similar to that of other species (reviewed in [5]). Since the dog is commonly used as an animal model of human dermatologic diseases, these cultures should be useful for comparative in vitro studies of the pathogenesis of a variety of diseases. Various aspects of the comparative cell biology and biochemistry of canine keratinocyte cultures are currently under investigation to more clearly establish the model.

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